An adenosine nucleoside inhibitor of dengue virus

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Dengue virus (DENV), a mosquito-borne flavivirus, is a major public health threat. The virus poses risk to 2.5 billion people worldwide and causes 50 to 100 million human infections each year. Neither a vaccine nor an antiviral therapy is currently available for prevention and treatment of DENV infection. Here, we report a previously undescribed adenosine analog, NITD008, that potently inhibits DENV both in vitro and in vivo. In addition to the 4 serotypes of DENV, NITD008 inhibits other flaviviruses, including West Nile virus, yellow fever virus, and Powassan virus. The compound also suppresses hepatitis C virus, but it does not inhibit nonflaviviruses, such as Western equine encephalitis virus and vesicular stomatitis virus. A triphosphate form of NITD008 directly inhibits the RNA-dependent RNA polymerase activity of DENV, indicating that the compound functions as a chain terminator during viral RNA synthesis. NITD008 has good in vivo pharmacokinetic properties and is biologically available through oral administration. Treatment of DENV-infected mice with NITD008 suppressed peak viremia, reduced cytokine elevation, and completely prevented the infected mice from death. No observed adverse effect level (NOAEL) was achieved when rats were orally dosed with NITD008 at 50 mg/kg daily for 1 week. However, NOAEL could not be accomplished when rats and dogs were dosed daily for 2 weeks. Nevertheless, our results have proved the concept that a nucleoside inhibitor could be developed for potential treatment of flavivirus infections.

antiviral therapy | flavivirus | viral replication

The family Flaviviridae includes 3 genera: *Flaviviruses*, *Pestiviruses*, and *Hepacivirus*. Many members from the genus *Flavivirus* are arthropod-borne and cause significant human diseases, such as the 4 serotypes of dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV) (1). Human vaccines are currently available only for YFV, JEV, and TBEV. Development of a vaccine for DENV has been challenging, principally because of the need to immunize and induce long-lasting protection against all 4 serotypes of DENV simultaneously; an incompletely immunized individual may be sensitized to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), either of which is a life-threatening disease (2). These complications have underscored the importance for development of an effective therapy for DENV and other flavivirus infections.

The genome of flavivirus is a single-stranded plus-sense RNA of about 11,000 nucleotides in length. The single ORF of the viral genome encodes a polyprotein, which consists of 3 structural proteins (capsid, premembrane or membrane, and envelope) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with membranes from host cells, form viral particles. The NS proteins play multiple roles, including viral RNA replication, virion assembly (3–5), and evasion of host immune response (6–10). Two nonstructural proteins have enzymatic activities: NS3 functions as a protease (with NS2B as a cofactor), a nucleotide triphosphatase, a 5′-RNA triphosphatase, and a helicase, and NS5 acts as a methyltransferase and an RNA-dependent RNA polymerase (RdRp) (11). NS3 and NS5 are 2 key components of viral replication complex and have been the preferred targets for antiviral development.

In this paper, we report an adenosine analog that selectively inhibits flaviviruses. A triphosphate form of the adenosine analog directly inhibits the recombinant RdRp of DENV, through termination of RNA chain synthesis. Importantly, the nucleoside inhibitor is available orally (p.o.) and has good in vivo pharmacokinetic properties. Using dengue mouse models, we show that the compound suppressed peak viremia, reduced cytokine elevation, and completely prevented infected mice from death.

Results and Discussion

Identification of NITD008 As a Potent Inhibitor of DENV. We took an adenosine-based nucleoside approach to develop inhibitors of DENV RdRp. Analysis of over 90 previously undescribed adenosine analogs identified compound NITD008 (Fig. 1A) with potent antiviral activity. Compared with adenosine, NITD008 contains a carbon substitution for N-7 of the purine and an acetylene at the 2′ position of ribose. The compound (up to 50 μM) is not cytotoxic in Vero (Fig. 1B), HEK 293, Huh-7, HepG2, A549, and BHK-21 cell lines or in primary human peripheral blood mononuclear cells (PBMCs) [supporting information (SI) Fig. S1]. NITD008 inhibited DENV-2 in a dose-responsive manner, with an EC_{50} value (the compound concentration required to inhibit 50% of viral titer) of 0.64 μM; treatment with 9 μM compound reduced viral titer by $>10^4$-fold (Fig. 1C). In addition to DENV-2, NITD008 potently inhibited the 3 other serotypes of DENV (including clinical isolates) when tested on different cell lines (BHK-21, A549, and Huh-7) and on human PBMCs (Table S1).
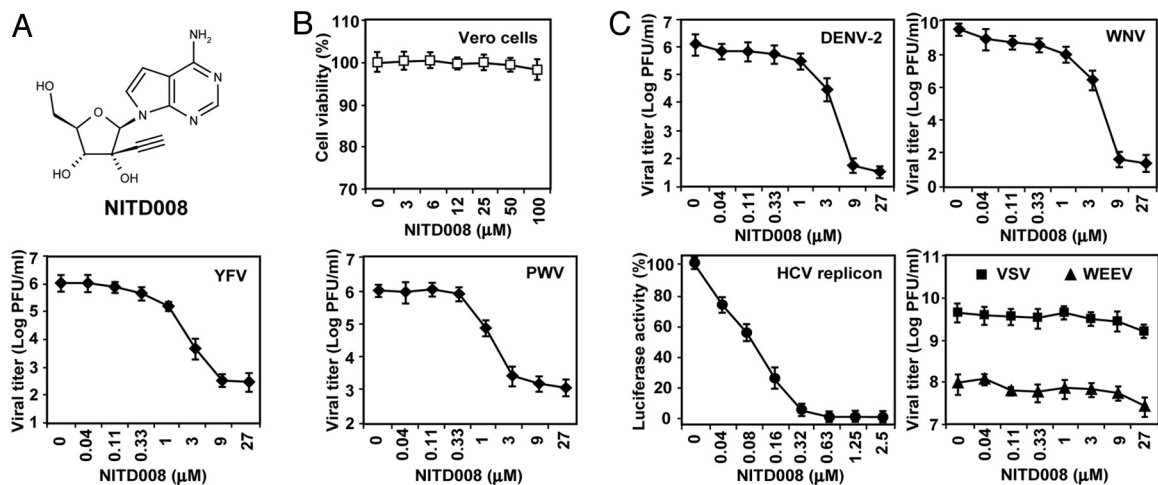
Fig. 1. Antiviral activity of NITD008. (*A*) Structure of NITD008. (*B*) Cytotoxicity in Vero cells. Vero cells were incubated with NITD008 at the indicated concentrations for 48 h. Cell viability was measured using a 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay and presented as a percentage of colorimetric absorbance derived from the compound-treated cells compared with that from the mock-treated cells. (*C*) Antiviral spectrum. Vero cells were infected with indicated viruses at a multiplicity of infection of 0.1 and treated immediately with NITD008. For DENV-2, WNV, YFV, PWV, and WEEV, culture medium was collected at 48 h postinfection and measured for viral titers using plaque assays. For VSV, culture medium was collected at 16 h postinfection and measured for viral titer. For HCV, Huh-7 cells carrying a luciferase replicon of HCV (19) were incubated with NITD008 and assayed for luciferase activity at 48 h posttreatment; relative luciferase activities were presented with the mock-treated replicon cells set as 100%. Average results and SDs ($n = 3$) are presented.

NITD008 Selectively Inhibits Flaviviruses. To examine the antiviral spectrum, we performed viral titer reduction assays using other flaviviruses. WNV, YFV, and Powassan virus (PWV) were selected as respective members from the JE, YF, and TBE serocomplexes within the genus *Flavivirus*. As shown in Fig. 1*C*, NITD008 inhibited all 3 flaviviruses; treatment with 9 μM compound suppressed viral titers by 10^3-10^6-fold. Remarkably, the compound also inhibited a luciferase-reporting replicon of hepatitis C virus (HCV, genotype 1b), a member from the genus *Hepacivirus*, with an EC_{50} value of 0.11 μM. In contrast, NITD008 did not inhibit Western equine encephalitis virus (WEEV, a plus-strand RNA alphavirus) and vesicular stomatitis virus (VSV, a negative-strand RNA rhabdovirus). These results demonstrate that NITD008 specifically inhibits viruses within the family *Flaviviridae*.

Effect of Human Plasma Proteins on Antiviral Potency of NITD008. We examined the effect of human plasma proteins on the efficacy of NITD008 against DENV. Addition of human serum proteins, human serum albumin (40 mg/mL), and α_1-acid glycoprotein (2 mg/mL) increased the EC_{50} value by 2–3-fold (Table S2). Direct binding analysis showed that 52% and 56% of NITD008 was bound to rat and human plasma proteins, respectively (Fig. S2), suggesting that the shift in EC_{50} value may result from the plasma protein binding of the compound. Nevertheless, the EC_{50} shift does not significantly affect the in vivo efficacy (see below).

NITD008 Inhibits DENV RdRp Through Termination of RNA Chain Synthesis. Both biochemical and genetic approaches were used to analyze the mechanism of action of NITD008. The biochemical approach used a primer extension-based RdRp assay; the assay measures the incorporation of [α-^{33}P]GTP in the presence of cold GTP and ATP using an RNA template composed of a U followed by a (C)$_{19}$-track (Fig. 2*A*). A triphosphate derivative of NITD008 (ppp-NITD008) was chemically synthesized to serve as an RdRp substrate. In the absence of ppp-NITD008, a set of short RNA species and a fully extended RNA product were detected on a denaturing polyacrylamide gel (Fig. 2*B*, lane 1). Addition of ppp-NITD008 to the RdRp reaction reduced the amounts of RNA products in a dose-dependent manner (Fig. 2*B*, lanes 3 and 6–20). Quantification of the RNA products esti-

mated an IC_{50} value of 0.31 μM (Fig. 2*C*). As controls, addition of 3′-deoxy ATP (3′d-ATP; Fig. 2*B*, lanes 2, 4, and 5) and 3′-deoxy GTP (3′d-GTP; Fig. 2*B*, lane 4), 2 known RNA chain terminators, suppressed RNA synthesis. These results suggest that ppp-NITD008 acts on viral RdRp to terminate RNA synthesis. For the genetic approach, we attempted to select
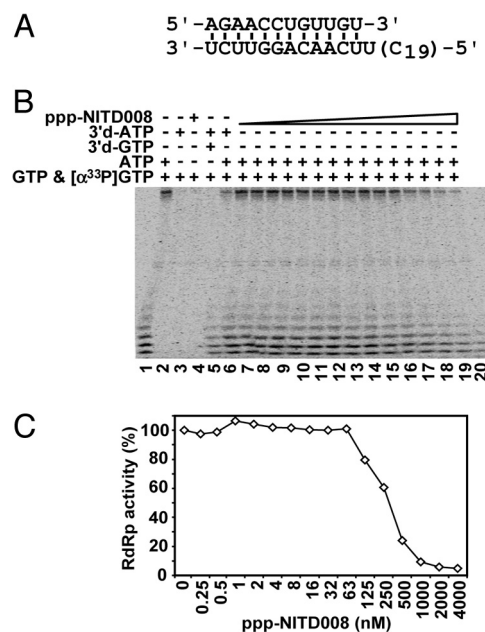
Fig. 2. Inhibition of RdRp activity by NITD008 triphosphate. (*A*) RNA template for RdRp assay. The template RNA contains the authentic initial 12 nucleotides of the DENV-2 minus-sense RNA, followed by a U and a (C)$_{19}$-track. (*B*) RdRp primer extension assay. The RdRp assays contained 500 nM RNA template, 100 nM DENV-2 NS5, 5 μM cold GTP, and 1 μCi [α^{33}P]GTP. ATP (0.25 μM), 3′d-ATP (1 μM), 3′d-GTP (1 μM), or ppp-NITD008 (1 μM for lane 3 and 0.25 nM–4 μM for lanes 6–20 with a 2-fold increment of compound concentration) was added to the RdRp reaction, as indicated. The RdRp products were analyzed on a polyacrylamide denaturing gel. (*C*) Quantification of the RdRp results from *B* using a PhosphorImager.

Table 1. Pharmacokinetic (PK) parameters of NITD008 following i.v. and p.o. dosing to mouse, rat, and dog

Species	PK parameters (p.o. dosing)*					PK parameters (i.v. dosing)†		
	Dose (mg/kg)	C_{max} (μM)	T_{max} (h)	AUC (μM*h)	F (%)	V_{ss} (L/kg)	CL (mL·min^{-1}·kg^{-1})	Elim. $T_{1/2}$ (h)
Mouse	25	3.00	0.5	22.32	48	3.71	31.11	4.99
Rat	28.4	4.10	2.0	30.35	68	13.37	36.37	9
	106.5	12.26	0.5	110.24	66	—	—	—
	272.4	14.30	0.5	211.45	49	—	—	—
Dog	5	3.93	1–2	42.37	61	5.05	4.13	16–32

*The p.o. PK parameters were obtained by dosing animals with the compound in the formulation described in the text. AUC, area under the curve ($t = 0$ to infinite); C_{max}, maximal plasma concentration; F: absolute oral bioavailability; T_{max}, time of peak plasma concentration.

†The i.v. PK parameters for the mouse and rat were obtained by dosing animals i.v. with 5 mg/kg in formulation containing ethanol/PEG400/D5W (5% [wt/vol] dextrose in water) in a ratio of 10:30:60. The i.v. PK parameters for the dog were obtained by dosing animals with 1 mg/kg in the formulation described in the text. CL, clearance from plasma after i.v. bolus administration; Elim. $t_{1/2}$, terminal half-life for elimination (noncompartmental estimate); V_{ss}, volume of distribution at steady state.

resistant viruses. No resistant virus was recovered after continuous culturing of DENV or WNV on BHK-21 or Vero cells at various compound concentrations for up to 4 months. The results indicate that viruses resistant to NITD008 do not readily emerge in cell culture.

NITD008 Is Orally Bioavailable and Has Good Pharmacokinetic Properties. For preparation of an in vivo efficacy study, we determined the pharmacokinetic parameters of NITD008 in mice (Table 1). Comparison of different formulation recipes showed that NITD008 exhibited the best pharmacokinetic parameters when formulated using 6 N of HCl (1.5 equimolar amount), 1 N of NaOH (pH adjusted to 3.5), and 100 mM citrate buffer (pH 3.5). Following i.v. injection, the compound had a high volume of distribution (3.71 L/kg) and a low systemic clearance (31.11 mL·min^{-1}·kg^{-1}), resulting in a long elimination half-life ($t_{1/2}$ = 4.99 h). After p.o. dosing, the compound was rapidly absorbed (time of peak plasma concentration = 0.5 h), with a maximal plasma concentration of 3 μM and bioavailability of 48%. Desirable pharmacokinetic parameters were also obtained for the compound in rats and dogs (Table 1). Overall, the results indicate that NITD008 has a good in vivo pharmacokinetic property and is biologically available through p.o. administration.

In Vivo Efficacy of NITD008 in Dengue Mouse Models. A dengue viremia model in mice (12) was used to examine the in vivo efficacy of NITD008. Infection of AG129 mice (lacking IFN-α/β and IFN-γ receptors) with DENV-2 (strain TSV01) leads to viremia (which peaks on day 3 postinfection) and cytokine elevation that are characteristic of dengue fever (DF). Starting immediately after infection, p.o. dosing of infected mice with NITD008 at 3, 10, 25, and 50 mg/kg twice daily reduced peak viremia by 1.8-, 5-, 10-, and 35-fold, respectively; the treatment also suppressed viral NS1 level in plasma by 1.5-, 3-, 6-, and 14-fold, respectively (Fig. 3A, *Middle*). Delayed start of treatment (at 25 mg/kg) up to 48 h postinfection reduced plasma viremia by 7–10-fold and, to a lesser extent, the NS1 level (Fig. 3A, *Bottom*).

We further tested NITD008 in a dengue lethal model in mice (13). Infection of AG129 mice with a mouse-adapted DENV-2 strain known as D2S10 causes inflammation and vascular leakage (the 2 hallmarks of DHF and DSS), leading to death within 5 days postinfection (13). Treatment of the mice immediately after viral infection with 1 mg/kg of NITD008 did not reduce mortality, but treatment with 3 mg/kg partially protected and treatment with $\geq$10 mg/kg completely protected the infected mice from death (Fig. 3B, *Top*). The peak viremia on day 3 postinfection did not change significantly when mice were treated with 1 mg/kg of NITD008 ($P = 0.23$, Student's t test), but

the viremia was suppressed by 1.6-, 4.8-, 6.4-, and 6.0-fold when mice were treated with 3, 10, 25, and 50 mg/kg of NITD008, respectively ($P < 0.05$; Fig. 3B, *Middle*). Delayed start of treatment (at 25 mg/kg) at 24 h postinfection significantly protected the infected mice from death; however, start of treatment at 48 h postinfection (thus, each mouse received only 2 doses of compound treatment) did not substantially improve the mortality rate (Fig. S3).

Inflammatory cytokines are increased in the blood of patients who have DENV (14). Cytokines, such as TNF and IL-6, play critical roles in vascular leakage, leading to DSS (14). In the lethal mouse model, elevations of both TNF and IL-6 (but not IFN-γ) were mitigated on treatment with $\geq$10 mg/kg of NITD008 (Fig. 3B, *Bottom*). Taken together, the results demonstrate that NITD008 can suppress peak viremia, decrease cytokine elevation, and prevent death in vivo, even when the start of treatment is delayed after viral infection.

In Vitro and in Vivo Toxicity and Safety. We examined the in vitro and in vivo toxicity of NITD008. The in vitro toxicity was assessed in more than 150 biochemical assays, including the Ames test for genotoxicity, hERG (the human ether-a-go-go related gene) channel for cardiovascular toxicity, CYP450 inhibition for drug–drug interaction, micronucleus assay for mutagenicity, and various receptors, ion channels, and kinase profiles. The compound did not show inhibition in any of these assays (Table S3 and Table S4). In vivo toxicity analysis showed that no observed adverse effect level (NOAEL) could be achieved when rats were dosed p.o. with NITD008 at 50 mg/kg daily for 1 week. However, NOAEL could not be accomplished when rats (10 mg/kg/day) and dogs (1 mg/kg/day) were dosed daily for 2 weeks. During the second week of treatment, the dogs showed moderate weight loss, decreased motor activity, retching, and feces with mucoid or blood and the rats showed irreversible corneal opacities, blood abnormalities, and movement disorders. Experiments are ongoing to investigate the cause of toxicity and to overcome the side effects. Because DF is an acute disease with fever duration of less than 1 week (1), the length of therapeutical treatment is expected to be less than 6 days. This leads to the question of whether such a short treatment period would cause adverse effects in humans. In support of this argument, none of the mice showed any side effects during the 3-day compound treatment in the in vivo efficacy experiments (Fig. 3).

One goal of anti-DENV therapy is to prevent patients with DF from developing DHF and DSS. Prospective studies of clinically characterized patients indicated that plasma levels of viremia and viral NS1 were 10–100-fold higher in patients with DHF/DSS than in those with DF (15). In agreement with these clinical findings, our in vivo efficacy results demonstrate that treatment of DENV-infected mice with NITD008 at $\geq$10 mg/kg reduced

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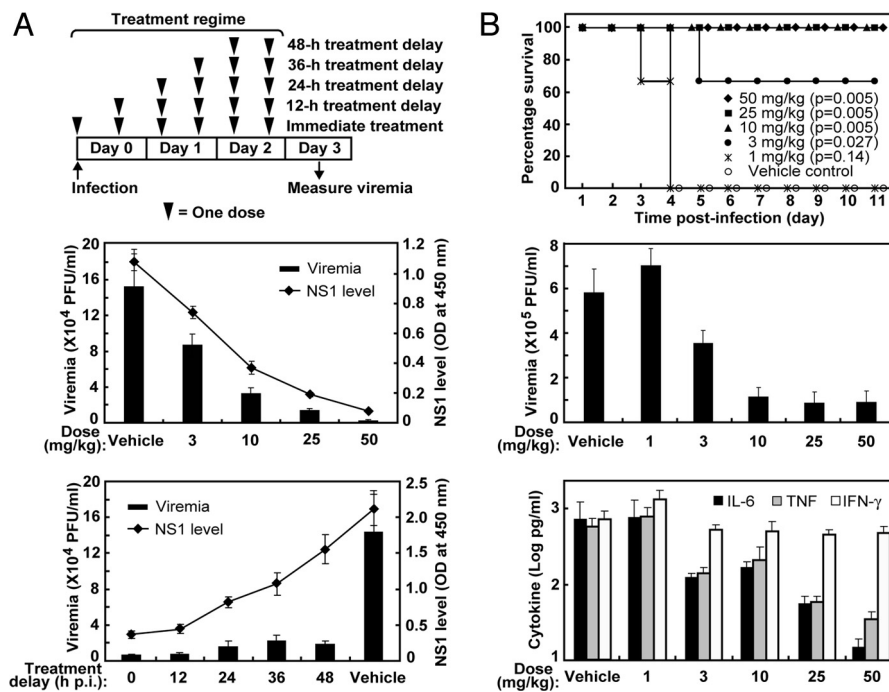
Fig. 3. In vivo efficacy of NITD008. (*A*) Viremia mouse model (12). AG129 mice (defective in IFN-α/β and IFN-γ receptors) were inoculated i.p. with 2 × 10⁶ pfu DENV-2 (strain TSV01) on day 0. (*Top*) Depiction of different treatment regimens. The mice (6 or 8 animal per group) were dosed p.o. with NITD008 twice a day. The peak viremia on day 3 postinfection (p.i.) was quantified by plaque assay; viral NS1 level in serum was measured by ELISA (12). (*Middle*) Indication of viremia and NS1 levels for the mice with immediate treatment after infection. (*Bottom*) Viremia and NS1 level from the mice with delayed start of treatment after infection (dosed at 25 mg/kg). (*B*) Lethal mouse model (13). AG129 mice were inoculated i.v. with 3 × 10⁷ pfu/mL DENV-2 strain D2S10 (13) and immediately treated with the indicated amount of NITD008 twice daily (6 mice per group). (*Top*) Survival curve. (*Middle*) Peak viremia. (*Bottom*) Summary of cytokine levels in serum.

peak viremia by >4.8-fold, leading to complete protection of infected mice from death. Compared with the compounds that were previously reported to have in vivo antiviral activities, NITD008 (EC₅₀ = 0.64 μM) showed better potency in cell culture than castanospermine (an inhibitor of host α-glucosi-dase, EC₅₀ range: 1–86 μM depending on cell type) (16) and 7-deaza-2′-C-methyl-adenosine (a nucleoside inhibitor, EC₅₀ = 15 μM) (17). Taken together, the current study has proved the concept that a nucleoside inhibitor can be developed for clinical treatment of DENV infection.

Methods

In Vitro Antiviral Assays. The synthesis of NITD008 and its triphosphate derivative (ppp-NITD008) will be described elsewhere. For measurement of compound cytotoxicity, cells (1 × 10⁴ cells per well of a 96-well plate) were incubated with various concentrations of NITD008 for 48 h; cell viability was quantified using a 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bro-mide assay according to the manufacturer's protocol (American Type Culture Collection). A viral titer reduction assay was performed to measure the anti-viral efficacy of NITD008 in cell culture. Briefly, Vero cells were seeded in a 12-well plate (4 × 10⁵ cells per well). At 24 h postseeding, the cells were infected with indicated viruses at a multiplicity of infection (MOI) of 0.1 and treated immediately with NITD008. For DENV-2 (New Guinea C), WNV (New York 3356), YFV (17D vaccine strain), PWV (strain 64–7062), and WEEV (strain Cova 746), culture medium was collected at 48 h postinfection. For VSV (New Jersey serotype), culture medium was collected at 16 h postinfection. Viral titers of all samples were determined by plaque assays, as previously described (18). For HCV, Huh-7 cells harboring a luciferase-reporting replicon of geno-type 1b (con1) HCV (19) were incubated with NITD008 and assayed for lucif-erase activity at 48 h posttreatment. In addition to a plaque assay, a cell-based flavivirus immunodetection assay and a CellTiter-Glo luminescent cell viability assay (Promega) were performed to measure the antiviral activity of NITD008, as detailed in the notes to Table S1.

For the RdRp assay, 2 synthetic RNAs, representing the primer strand and the template strand (Fig. 2*A*), were annealed by incubating at 80 °C for 1 min, followed by cooling down to room temperature for 30 min. The annealing

mixture contained 50 mM Tris (pH 8.0), 100 mM NaCl, and 50 μM synthetic RNA. The RdRp reactions contained 500 nM annealed RNA template, 100 nM full-length NS5 of DENV-2, 5 μM cold GTP, and 1 μCi of [α³³P]GTP. The reactions were incubated at 30 °C for 1 h. ATP, 3′d-ATP, 3′d-GTP, or ppp-NITD008 was added to the RdRp reaction, as indicated in the legend to Fig. 2*B*. After separating the reactions on an 18% polyacrylamide (wt/vol) denaturing gel, the RNA products were quantified using a PhosphorImager (GE Health-care).

Antiviral Efficacy in Dengue Mouse Models. The in vivo efficacy of NITD008 was evaluated in a dengue viremia model and a lethal model in mice. Both models use AG129 mice (with knockout IFN-α/β and IFN-γ receptors), purchased from B & K Universal. DENV-2 strains TSV01 (12) and D2S10 (13), respectively, were used in the 2 models and were propagated in C6/36 mosquito cells grown in RPMI-1640 medium with 5% FBS (vol/vol) at 28 °C. The evaluation of inhibitors in the viremia model (including the measurements of virus and cytokines in blood) was performed as previously reported (12). The level of NS1 was quantified using a BioRad NS1 ELISA kit. The evaluation in the lethal model was performed by injecting mice i.v. with 0.2 mL of RPMI-1640 medium containing 3 × 10⁷ pfu/mL DENV-2 strain D2S10; the infected mice were then subjected to different treatment regimens, as indicated in each experiment. NITD008 in 0.2–0.25 mL of formulation solution (described in *Results*) was administered by p.o. gavage. The mice (6 or 8 mice per group) were monitored twice a day. Statistical analysis was performed by the log-rank test and Student's *t* test using SigmaPlot/SigmaStat software (Systat Software Inc.).

Pharmacokinetic Analysis. Female CD-1 mice, female Wistar rats, and male beagle dogs were used to analyze the in vivo pharmacokinetic parameters. The dosing scale and formulation are detailed in individual experiments. For mice, blood samples were collected at 0.02 (i.v. only), 0.08 (p.o. only), 0.25, 0.5, 1, 2, 4, 6, 8, 16, and 24 h postdosing. For rats, blood samples were collected at 0.17, 0.5, 1, 2, 4, 6, 8, 17, 24, 32, and 48 h postdosing. For dogs, blood samples were collected at 0.083 (i.v. only), 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h postdosing. At each time point, terminal samplings were obtained from 3 mice (for both i.v. and p.o. routes) and serial samplings were obtained from 3 rats (for both i.v. and p.o. routes), 2 dogs for the i.v. route, and 3 dogs for the p.o. route. The plasma concentrations of NITD008 were measured using LC/MS/MS. Pharma-cokinetic parameters were calculated by a noncompartmental approach using

1. Gubler D, Kuno G, Markoff L (2007) Flaviviruses. *Fields Virology*, eds Knipe DM, Howley PM (Lippincott Williams & Wilkins, Philadelphia) 5th Ed, Vol 1, pp 1153–1253.
2. Whitehead SS, Blaney JE, Durbin AP, Murphy BR (2007) Prospects for a dengue virus vaccine. *Nat Rev Microbiol* 5(7):518–528.
3. Kummerer BM, Rice CM (2002) Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. *J Virol* 76:4773–4784.
4. Liu WJ, Chen HB, Khromykh AA (2003) Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. *J Virol* 77:7804–7813.
5. Patkar CG, Kuhn RJ (2008) Yellow Fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions. *J Virol* 82:3342–3352.
6. Best SM, et al. (2005) Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist. *J Virol* 79:12828–12839.
7. Guo J, Hayashi J, Seeger C (2005) West Nile virus inhibits the signal transduction pathway of alpha interferon. *J Virol* 79:1343–1350.
8. Liu W, et al. (2005) Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. *J Virol* 79:1934–1942.
9. Munoz-Jordan JL, et al. (2005) Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J Virol* 79:8004–8013.
10. Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A (2003) Inhibition of interferon signaling by dengue virus. *Proc Natl Acad Sci USA* 100:14333–14338.
11. Lindenbach BD, Thiel H-J, Rice CM (2007) Flaviviridae: The virus and their replication. *Fields Virology*, eds Knipe DM, Howley PM (Lippincott William & Wilkins, Philadelphia), 5th Ed, Vol 1, pp 1101–1152.
12. Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG (2007) A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. *J Infect Dis* 195:665–674.
13. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E (2006) Murine model for dengue virus-induced lethal disease with increased vascular permeability. *J Virol* 80:10208–10217.
14. Halstead SB (1988) Pathogenesis of dengue: Challenges to molecular biology. *Science* 239:476–481.
15. Libraty DH, et al. (2002) High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J Infect Dis* 186:1165–1168.
16. Whitby K, et al. (2005) Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. *J Virol* 79:8698–8706.
17. Olsen DB, et al. (2004) A 7-deaza-adenosine analog is a potent and selective inhibitor of hepatitis C virus replication with excellent pharmacokinetic properties. *Antimicrob Agents Chemother* 48:3944–3953.
18. Puig-Basagoiti F, et al. (2006) Triaryl pyrazoline compound inhibits flavivirus RNA replication. *Antimicrob Agents Chemother* 50:1320–1329.
19. Krieger N, Lohmann V, Bartenschlager R (2001) Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* 75:4614–4624.

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